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Two sodium-channel genes in *Drosophila*: Implications for channel diversity

(ion channel/neurogenetics/evolution)

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ABSTRACT **We describe two *Drosophila melanogaster* transcription units that are highly homologous to a rat Na^+-channel cDNA. They appear to encode the major subunits of two distinct Na^+-channel proteins. One of these maps to the second chromosome and is identical to a Na^+-channel gene whose partial sequence has been previously reported [Salkoff, L., Butler, A., Wei, A., Scavarda, N., Giffen, K., Ifune, K., Goodman, R. & Mandel, G. (1987) *Science* 237, 744–749]. The other transcription unit maps to position 14C/D, on the X chromosome, close to the paralyzed (*para*) gene. Mutations in *para* affect membrane excitability in *Drosophila* neurons [Ganetzky, B. & Wu, C. F. (1986) *Annu. Rev. Genet.* 20, 13–44]. Sequence comparisons suggest that two Na^+-channel genes arose early in evolution, before the divergence of vertebrate and invertebrate lines.**

Ion channels are a diverse group of integral membrane proteins regulating the passage of ions through cell membranes. Electrophysiological, pharmacological, and biochemical methods have distinguished several types of sodium and calcium channels generally involved in the depolarization of excitable membranes (1–3). An even larger number of distinct potassium channel types involved in membrane repolarization has also been detected (4). The differential distribution of these channel types underlies the range of electrical responses in neurons (5).

The cloning of ion-channel genes allows the detailed biochemical and biophysical characterization of channel proteins. Recent results have shown that voltage-gated channels share several sequence motifs (2, 6–15). Na^+ channels contain four internal repeats about 250 residues in size that are approximately 50% identical to one another. These repeats are also called homology domains. In each domain multiple hydrophobic segments called S1, S2, S3, S5, and S6 flank a positively charged segment called S4. S4 segments consist of four to eight iterations of a 3-residue sequence, Arg-Xaa-Xaa, where Xaa is a hydrophobic residue and lysine is sometimes substituted for arginine. A putative Ca^{2+} channel has an identical structural organization (15). All K^+ channels cloned so far are similar in structure to a single homology domain of the Na^+ channel. The data suggest that these different channel types may have evolved from a single ancestral voltage-gated channel that arose early in phylogeny.

The isolation of ion-channel genes in an organism such as *Drosophila*, where genetic manipulations are feasible, facilitates the identification of other genes involved in ion-channel function. Several mutations affecting neural excitability have been identified in *Drosophila* (16, 17). Mutations that affect specific classes of K^+ channels (*Sh, eag, slo,* and *Hk*) cause abnormalities associated with increased membrane excitability (17). Conversely, mutations that affect Na^+ currents (*para*^{ts}, *nap*^{ts}, and *tip-E*) cause an overall decrease in membrane excitability (16–19). These mutations might identify structural genes for ion channels or genes for proteins involved in the synthesis, membrane distribution, or modulation of ion channels.

We have searched *Drosophila melanogaster* genomic libraries for sequences similar to an mRNA for the large subunit of a voltage-gated Na^+ channel from rat brain, with goals to (*i*) complement genetic approaches, (*ii*) analyze the diversity of Na^+-channel genes, and (*iii*) further understand the structure and evolution of ion channels. In this paper, we report the partial characterization of two transcription units that appear to encode distinct Na^+-channel proteins.* One of these genes probably corresponds to the *para* locus.

MATERIALS AND METHODS

Standard Techniques. Standard methods of molecular biology were as described in laboratory manuals (20, 21). Radiolabeled DNA probes were synthesized using the random hexamer primer method (22). *Drosophila* genomic DNA preparations, *in situ* hybridizations to polytene chromosomes, and DNA sequence analysis were performed as described (9, 10, 23–25).

Isolation of *Drosophila* Genomic DNA Clones Homologous to a Rat Na^+-Channel Gene. Three cDNA clones encoding parts of the major subunit of the rat brain Na^+ channel, RatIIA (Fig. 1 and ref. 7), were used as hybridization probes to screen λ phage libraries of *Drosophila* genomic DNA. The libraries were constructed by C. A. Kamb (Caltech) (9). Rat Na^+-channel cDNAs were a gift from A. Goldin and N. Davidson (Caltech) and V. Auld and R. Dunn (University of Toronto). The rat cDNA clones AG141, NA2.2, and NA8.4 contained coding sequences for approximately 75% of RatIIA (Fig. 1 and ref. 7). The sequences most highly conserved between different Na^+ channels lie largely within the homology domains (2, 7, 8). NA2.2 contains sequences encoding domain A, and NA8.4 encodes domains C and D. AG141 contains less conserved sequences. The hybridizations were carried out at 25°C in 0.75 M NaCl/0.025 M Na_2HPO_4/0.001 M EDTA/0.1% $NaDodSO_4$/0.05 M Tris/denatured salmon sperm DNA (100 μg/ml)/50% (vol/vol) formamide/10% (wt/vol) dextran sulfate at pH 7.5. The filters were rinsed at room temperature in 0.3 M NaCl/0.03 M sodium citrate/0.1% $NaDodSO_4$ and washed for 30 min at 55°C in the same solution. No cross-hybridization was observed when the wash temperature was raised to 65°C. Forty-two recombinant phage clones were isolated. These were placed into 21 groups of nonoverlapping clones based on a comparison of their restriction maps, and on hybridization experiments in which DNA from all 42 clones was probed with restriction fragments purified from particular clones. In some cases the

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*The sequence discussed in this paper is being deposited in the EMBL/GenBank data base (accession no. J04508).

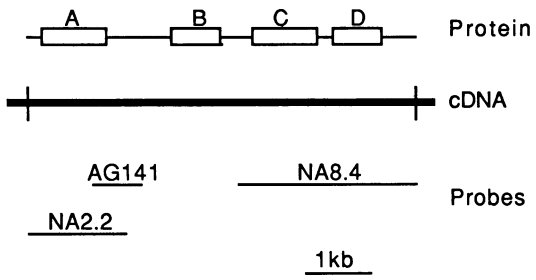

FIG. 1. Representations of the rat Na^+ channel RatIIA and the cDNA clones used as hybridization probes. The major subunit of RatIIA is a 2000-amino acid polypeptide with four homology domains. In the figure the domains are labeled A, B, C, and D from amino to carboxyl terminus. The domains are presumed to assemble in the membrane as "pseudo subunits" around an ion-selective pore. The cDNA probes used in the experiments reported here are shown at their apposite positions on the channel gene. NA2.2 is a cDNA extending from nucleotide −10 to 1511 (7). NA8.4 is a cDNA extending from nucleotide 3361 to 5868. AG141 is a 558-base-pair cDNA beginning at nucleotide 980. kb, Kilobase.

assignments were verified by *in situ* hybridizations of cloned DNA to polytene chromosomes from larval salivary glands. The groups were named for a representative clone.

In a second screen, 5 of the 21 groups were identified as strong candidates for ion-channel genes. About 70% of single-copy DNA from *D. melanogaster* does not form stable hybrids with DNA from a distant species of *Drosophila*, *Drosophila virilis*, under conditions corresponding to approximately 60% homology (26). Only 5 groups (A20, A4.3, A3.13, A4.5, and A4.11) had specific restriction fragments (1.5–3.5 kilobases long) that cross-hybridized with both rat Na^+-channel cDNAs and *D. virilis* genomic DNA. Hence only these contained DNA sequences similar to the rat Na^+-channel gene that were also phylogenetically conserved. Two groups (A4.3 and A3.13) that cross-hybridized most strongly with the rat Na^+-channel cDNAs were chosen for further characterization. Both of these groups were initially isolated with the rat probe NA8.4 (Fig. 1). The order of restriction endonuclease cleavage sites in the cloned DNA was determined (Fig. 2). Restriction fragments that cross-hybridized with rat channel sequences were used to screen *Drosophila* cDNA libraries.

Screening cDNA Libraries. Complementary DNA clones from A4.3 and A3.13 were obtained from two adult head cDNA libraries (gifts from P. Salvaterra, City of Hope, and C. Zuker, University of California, San Diego). In all, 14 independent clones representing A4.3 and 10 representing A3.13 were identified. The cDNAs were subcloned into plasmid vectors (Bluescript and M13mp18) for further analysis.

RESULTS AND DISCUSSION

A4.3 Defines a *Drosophila* Na^+ Channel. The partial nucleotide sequence of a cDNA (B1) from A4.3 was determined. A comparison (data not shown) shows that B1 is about 59% identical in nucleotide sequence to a region of the rat Na^+-channel gene, RSC2 (2). This region encodes segments S4 and S5 and a portion of S3 from homology domain C of the rat channel (amino acid residues 1287–1364, ref. 2). The amino acid sequence deduced from B1 is identically contained in DSC, a putative *Drosophila* Na^+ channel whose partial sequence has been reported (amino acid residues 1080–1155, ref. 8). The nucleotide sequences of B1 and DSC are identical for approximately 240 nucleotides (27). It has not been clearly demonstrated that DSC encodes a functional Na^+-channel protein. However, the sequence data strongly support this hypothesis (8).

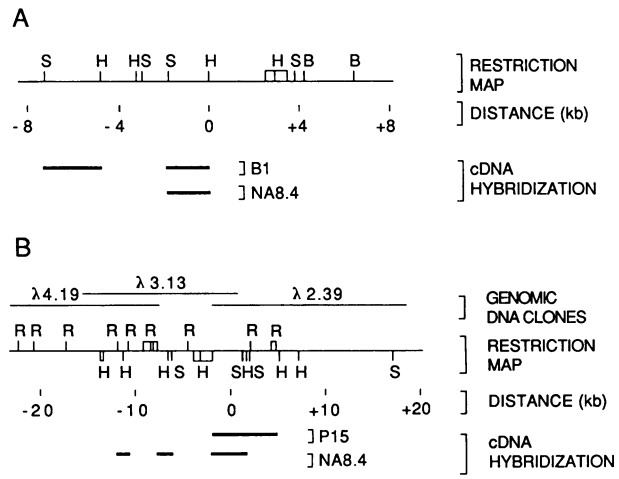

FIG. 2. Restriction maps of two genomic loci that appear to encode different *Drosophila* Na^+ channels. Distance is shown in kilobases of DNA. R, *Eco*RI; S, *Sal* I; B, *Bam*HI; H, *Hin*dIII. (*A*) Map of restriction enzyme cleavage sites in the genomic clone A4.3. The rat cDNA NA8.4 hybridizes to a 1.8-kilobase *Sal* I–*Hin*dIII fragment centered at about map position −1. The fragment was used as a hybridization probe to screen *D. melanogaster* cDNA libraries. The other rat cDNAs, NA2.2 and AG141, do not cross-hybridize to the DNA shown in the figure. The hybridization pattern of cDNA clone B1 is also indicated. The restriction map depicted here is similar to a portion of the DSC map (8) but there are some differences. It is possible that some restriction site polymorphisms exist between the two clones. (*B*) Map of restriction enzyme cleavage sites derived from eight genomic clones of the A3.13 group that were isolated using the rat cDNA probe NA8.4; three representative clones (A4.19, A3.13, and A2.39) are shown. Indicated are three noncontiguous restriction fragments that cross-hybridize with NA8.4 and with *D. virilis* genomic DNA. Each of these restriction fragments was used as a hybridization probe to screen *D. melanogaster* cDNA libraries. The hybridization pattern of cDNA P15 described in the text is also shown. The orientation of the gene based on sequence data and mapping of restriction enzyme cleavage sites is 5′ to 3′ from left to right.

The genomic clone A4.3 was mapped *in situ* on salivary gland chromosomes to a single site at 60E (see Fig. 4*A*). This location is identical to the reported cytogenetic map position of the DSC gene (8). Thus it appears that A4.3 and DSC define the same *Drosophila* gene. This conclusion is based on (*i*) the sequence identity we find between the two genes; (*ii*) the identical cytological map positions of A4.3 and DSC; and (*iii*) unique hybridization signals obtained with probes from A4.3 on blots of genomic DNA.

A3.13 Defines a Second *Drosophila* Na^+-Channel Gene. The order of restriction enzyme cleavage sites in genomic DNA around A3.13 is shown (Fig. 2*B*). Three noncontiguous restriction fragments in this DNA cross-hybridize with rat Na^+-channel cDNAs. Thus, at this level of analysis, it appears that homology to the rat Na^+-channel gene spans several kilobases of genomic DNA. We determined the nucleotide sequence of a cDNA (P15) from A3.13. The sequence (660 base pairs) is 68% identical to a region of RSC2 (2) that encodes homology domain D. A single open reading frame extends through the entire sequence of P15. The deduced amino acid sequence shows structural motifs characteristic of voltage-gated ion channels: an S4-like segment is flanked by three hydrophobic stretches of amino acid residues (Fig. 3). A majority of nucleotide differences between P15 and RSC2 are in "wobble base" positions and do not change the predicted polypeptides. Fig. 3 shows aligned amino acid sequences deduced from P15, RSC2, and DSC. A total of 141 residues are identical between rat and P15 sequences over the 212 residues shown, while 108 residues are identical between P15 and DSC. The S4-like segments of

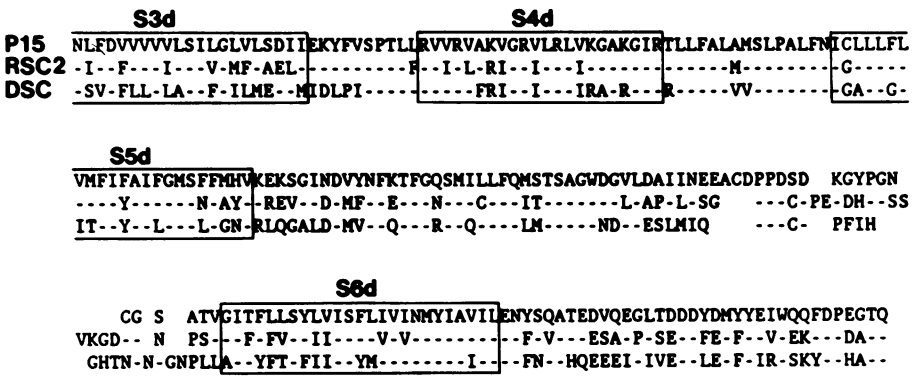

FIG. 3. Amino acid sequence derived from A3.13. The protein sequence deduced from the *Drosophila* cDNA P15 is depicted in the figure (using the single-letter amino acid code). The cDNA is incomplete as judged by the presence of a single unbroken open reading frame that extends through its entire length. For comparison, it is aligned with homologous sequences encoded in the rat Na^+ channel RSC2 (middle line) and in a putative *Drosophila* Na^+ channel, DSC (bottom line). Amino acid identities with P15 are indicated by dashes. Gaps are inserted for optimal alignment. Segments S3 to S6 of homology domain D of RSC2 are shown in boxes. For reference, the sequences shown in the figure are amino acids 1596–1812 of RSC2 (2) and amino acids 1383–1595 of DSC (8). The rat Na^+ channel RatIIA (7) is more than 99% identical to RSC2 (2). Nucleotide sequences are available on request.

all three sequences contain exactly eight positively charged residues; the hydrophobic segments are also highly conserved. Thus, the data strongly suggest that A3.13 encodes the major subunit of a *Drosophila* Na^+ channel.

A3.13 Probably Corresponds to the *para* Locus of *Drosophila*. *In situ* hybridizations to *Drosophila* polytene chromosomes show that the cytological location of A3.13 is 14C/D (Fig. 4*B*), virtually identical to the *para* locus that has been mapped to 14C6-8 (17). A chromosomal walk through *para* has included many chromosomal breakpoints that uncover *para* mutations and sequences of para cDNAs reveal homology to Na^+-channel genes (28, 29). DNA from A3.13 hybridizes to cloned DNA from this walk (K. Loughney and B. Ganetzky, personal communication). Thus, a comparison of results from the two laboratories indicates that A3.13 is derived from the *para* locus.

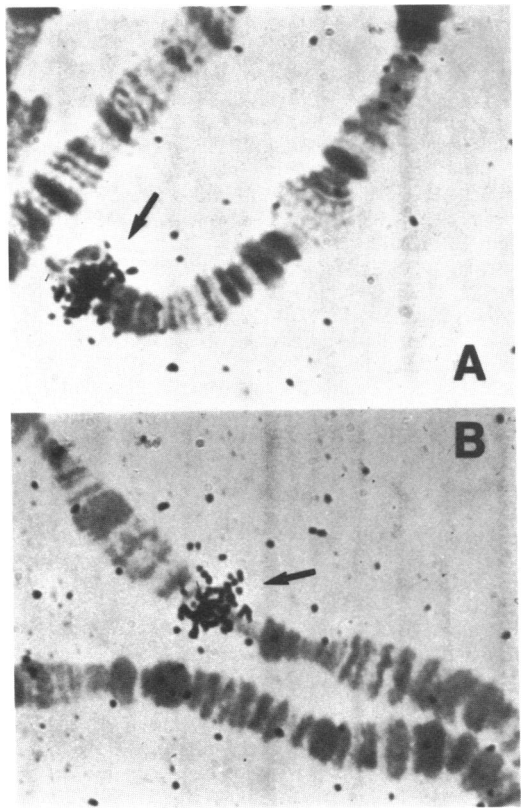

FIG. 4. Cytological mapping of *Drosophila* genomic clones by *in situ* hybridizations to polytene chromosomes. (*A*) Clone A4.3 maps to 60E at the tip of the right arm of chromosome 2, very close to the reported cytogenetic location of DSC. We are unaware of any neural excitability mutant in *Drosophila* that maps to this location. (*B*) The genomic clone A3.13 maps to the region between 14C and 14D on the X chromosome. Chromosome rearrangements that uncover mutations in *para* have been mapped to 14C6-8 (17).

Many lines of evidence have suggested that *para* could be a structural gene for a Na^+ channel. (*i*) Flies carrying one of several *para* alleles show rapid and reversible paralysis above characteristic restrictive temperatures (17, 30). (*ii*) Paralysis is associated with a temperature-dependent block in action potential propagation in some neurons (17, 31–33). (*iii*) Cultured neurons from $para^{ts}$ larvae show a temperature-dependent resistance to veratridine, a neurotoxin that binds and stabilizes Na^+ channels in an "open" conformation (34). (*iv*) Electrophysiological recordings from cultured neurons from *para* embryos show a significant reduction in the number of neurons expressing a Na^+ current (35). Thus, these lines of evidence strongly suggest that *para* encodes the major subunit of a *Drosophila* Na^+ channel.

Genes for two neural excitability mutants in *Drosophila* (*para* and Shaker), which were implicated in ion channel function, have now been cloned (9, 11, 13). Both appear to encode ion-channel proteins. This increases the likelihood that behavioral mutants with similar phenotypes identify additional ion-channel genes. These findings also increase interest in the molecular characterization of loci that genetically interact with Shaker and *para* (16, 17).

Implications for Na^+-Channel Evolution and Diversity. There is increasing evidence that vertebrate Na^+ channels constitute a diverse family of proteins (1). Electrophysiological and pharmacological methods have distinguished two classes of Na^+ current in rat skeletal muscle membranes and a slightly different current in rat brain (1). Distinctive Na^+ currents have been seen in Purkinje cells of guinea pigs (36) and in type I astrocytes in rat optic nerve (37). Na^+ channels in rat muscle membrane are different from those in T-tubular membranes (38, 39). In part this diversity may be due to the existence of multiple genes, as mRNAs for three distinct Na^+ channels are simultaneously expressed in rat brain (2, 7). The three proteins encoded by these transcripts are 95% identical to each other over the region depicted in Fig. 3. Table 1 shows sequence homologies between four putative Na^+-channel proteins. These figures have been computed for about 210 residues for which sequence is available for all the channels. The predicted protein sequence of *para* is 67% identical to the rat channel (RSC2) and 62% identical to the eel channel. It is,

Table 1. Percent homologies between ion channels

	% homology				
	para	DSC	RSC2	EELSCH	DHPR
para	—	51	67	62	30
DSC		—	54	56	29
RSC2			—	75	31
EELSCH				—	29

The homologies have been computed for about 210 amino acids for which sequence information is available for *para*. RSC2 represents a Na^+ channel from rat brain (2); EELSCH represents the Na^+ channel from electric eel (6); DHPR represents the dihydropyridine receptor from rabbit skeletal muscle that is postulated to be a voltage-gated Ca^{2+} channel (15); and DSC represents a putative *Drosophila* Na^+ channel (8). Identical amino acids are scored as 1 and all substitutions are scored as 0. An insertion or deletion is scored as a single substitution independent of its size. We believe it is unlikely that the results of our limited sequence comparisons will be drastically changed when more sequence from *para* becomes available. This is based on the observation that the relative homologies among RSC2, EELSCH, and DSC remain fairly constant in all four homology domains of the channel proteins (8). For reference, the sequences are amino acids 1386–1602 for EELSCH (6) and amino acids 1183–1415 for DHPR (15). The sequences for DSC and RSC2 are as in Fig. 3.

however, only 51% identical to DSC over the same sequence. The DSC sequence is 54% and 56% identical to the rat and eel channels, respectively. All four Na^+-channel proteins are about 30% identical to the dihydropyridine receptor that has been proposed to function as a voltage-gated Ca^{2+} channel (15). The striking sequence similarity among these genes suggests that they share a common evolutionary origin. Voltage-gated Ca^{2+} channels have been detected in protists, whereas voltage-gated Na^+ channels appear to have evolved more recently (5). Thus, the sequence data are consistent with an evolutionary scenario in which the first ancestral Na^+ channel arose by duplication of, and divergence from, a voltage-gated Ca^{2+} channel. The strong homology among the Na^+-channel genes in vertebrates and in *Drosophila*, suggests that voltage-gated Na^+ channels evolved before the divergence of vertebrates and invertebrates about 600 million years ago (8). The fact that *para* is more closely related to the known vertebrate channels than it is to DSC is easily explained if DSC and *para* diverged from each other before *para* diverged from the vertebrate Na^+ channels. Though our arguments are based on limited sequence information, it appears that two distinct Na^+-channel genes existed in the early Cambrian period, even before the divergence of vertebrates from invertebrates. On this basis one would predict that DSC might define a subfamily of vertebrate Na^+ channels that are yet to be molecularly identified.

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